Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 23, No. (1) : 2022 : 158-166

ISSN-0972-3005

http://doi.org/10.53550/AJMBES.2022.v24i01.026

OPTIMISATION *OF BREVIBACTERIUM, BACILLUS* AND *PSEUDOMONAS* SPECIES FOR PRODUCTION OF LIPASE USING TOBACCO SEED OIL AND OLIVE OIL AS SOLE CARBON SOURCES

[1]OMOLADE, O.A., [2]ORJI, F.A. AND [1]ADEBAJO, L.O.

[1]Department of Microbiology, Faculty of Science, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria
[2]Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, PMB 21023, Ikeja, Lagos Sate, Nigeria

(Received 8 November, 2021; Accepted 2 February, 2022)

Key word: *Lipase, Bacteria, Optimization, Cultural conditions, Tobacco seed oil*

Abstract– This study considered the optimization of cultural conditions for enhanced lipolytic enzyme activities amongst 4 bacterial Isolates. Soil samples from different oil-impact soil areas in Ogun –State-Nigeria were obtained with soil auger and transported the Biotechnology Laboratory of Federal Institute of Industrial Research Oshodi, Lagos – Nigeria. Soil was subjected to Microbiology studies such as Primary Isolation. Pure cultures obtained test using tributyrin agar, Lipase – hyper producing bacterial Isolates were identified using biochemical tests and Molecular biology tools (Polymerase chain reaction and gene sequencing) out of a total of 11 bacterial Isolates, 4 bacterial Isolates identified as hyper-pro-ducers were Isolate H (*Bavibacillus brevis* strain HK 544), Isolate A (*Pseudommas aeruinosa* strain WES 2), Isolate B (*Bcillus megaterium* strain WH 13), and Isolate F (*Bacillus subtilis* BS 01)changes in PH of culture/production medium showed that pH 7.0, favoured the Lipase production in *Bacillus subtilis* (Isolate F) and *Pseudomonas aeruginosa* (Isolate A) while pH values of 8.0 favoured Lipase production in Isolate H (*Brevibacillus brevis*). Variation in temperature showed that the 4 bacterial strain responded best to Mesophilic PH ranges from 25 ^{o}C- 40 ^{o}C. The optimal Nitrogen source as identified in the study was KNO_3 and NH_4NO_3 at higher concentrations (6 g/C). In comparison, Tobacco seed oil (TSO) competed favorably as carbon source with Olive oil as activities of Lipase from the 4 bacterial Isolates ranged from 8.0 – 28.0 units. The use of lesser known oil based carbon source like Tobacco seed oil has shown a lot of value addition in terms of commercial value.

INTRODUCTION

Microbial lipases were initially described by early worker's like Khan *et al*. (1967) as extra-cellular in nature and are inducible, in some cases, by the inclusion of lipid substrates in the growth media. The activity and stability of lipases may depend on culture conditions. If the organisms are grown at alkaline pH, the lipase produced may have an alkaline pH optimum (Andersson *et al*., 1979).

Lipases are classified as triacylglycerol acylhydrolase (EC 3.1. 1.3) and they belong to the family of hydrolases which act on carboxylic ester bonds. Their physiological role is to hydrolyze triacylglycrol to diglycerides, monoglycerides, fatty acids and glycerol (Pallavil *et al*., 2015). They are Serine hydrolases (Akimolo *et al*., 1999) and contain the consensus sequence G-X_1-S-X_2-G as the catalytic moiety, where G-glycine, S-serine, X_1-histidine and X_2-glutamic or aspartic acid (Svendsen *et al*., 1994). Lipases are reported to be monomeric proteins, having molecular weight in the range of 19-60kDa. Their physical properties mainly depend on factors such as the position of the fatty acid and its degree of unsaturation. Lipases are the most pliable biocatalyst and bring about a wide range of bioconversion reactions, such as hydrolysis, inter-esterification, esterification, alcoholysis, acidolysis and aminolysis. They can act on a variety of substrates including natural oils, synthetic

triglycerides and esters of fatty acids. They constitute one of the most important group of biocatalyst for biotechnological applications (Javed *et al.*, 2017). Lipase-catalyzed processes generally offer cost-effectiveness as against the traditional downstream processing having the problems of energy consumption and toxic by-products (Moses *et al.*, 2020). Microbial lipases are of special interest because of their stability in organic solvents and their lack of requirements for co-factors, their broad substrate specification and high enantio-selectivity (Godfrey and West, 1996).

Bacterial lipases can be obtained by submerged fermentation (SmF) as well as solid-state fermentation (SSF). Due to easy production along with inexpensive techniques of fermentation and low energy consumption, the lipases obtained from microbes (fungi, yeast and bacteria) are given preference for industrial application (Mazhar *et al.*, 2017).

The global enzyme market was valued at $ 1,371.03 million in 2014 and is projected to grow cumulatively at 7.3% from 2015-2023 (Research and Market, 2015). The applications of Lipase in the industries are not limited to applications in organic chemical processing, detergent formulations, biosurfactants production, the dairy industry, the agrochemical industry, paper manufacture, nutrition/food industry, cosmetics, pharmaceutical processing and the development of lipase-based technologies for the synthesis of novel compounds (Gupta and Soni 2000; Musa and Adebayo-Tayo, 2012).

In this present study, eleven (11) lipase producing bacterial cultures were isolated from vegetable oil impacted soil in Ogun State-Nigeria and further subjected to lipase screening. Generally, high productivity of lipase has been achieved by culture medium optimization. Physicochemical factors such as pH, temperature were studied to check how the bacterial isolates responded to their variation using microbial lipase activities for response monitoring. Tobacco Seed Oil and Olive oil were the carbon sources studied for their influence on Lipase activities of the bacteria.

MATERIALS AND METHODS

Soil Sample collection

Soil samples from the dumpsite at various spent oil environment/soil (Sabo/kara market, mechanic village Ileshan, Eperu market dumpsite, Robo Market (Lafenwa), OOU palm oil processing plant site) all in Ogun State Nigeria were collected with the aid of a disinfected soil trowel, asoil auger. The soil from the above mentioned sites using four sterile universal bottles with lids labelled A, B, C and D, E respectively. The soil samples were collected in order to isolate and characterize the microorganisms that are lipolytic.

Primary isolation on Nutrient Agar

Twenty eight grams (28 g) of the agar was suspended in 1 L of distilled water. It was mixed to allow the agar to be suspended and left to stand until the mixture was uniform. Heat was applied with gentle agitation and boiled for one or two minutes until it was completely suspended. It was sterilized in the autoclave at 121 ^{o}C for 15 min (1.03 kg/sec^2). Total heterotrophic bacterial count present in the soil samples were determined using spread plate technique. Soil suspensions were prepared by 10 fold serial dilutions with 1 g of soil, using peptone water as diluents. 0.1 ml aliquots of appropriate dilutions were spread on triplicates of sterile nutrient agar. The plates were incubated for period of 18-24 hours in the incubator at 28^{o}C ± 2 ^{o}C Colonies that formed during this incubation period was counted using the formula;

$$\frac{\text{No. of colonies X dilution factor}}{\text{Amount used}}$$

Values expressed as cfug^{-1}. Enumeration of total heterotrophic bacteria was carried out using the procedures stated above. These procedures have been previously reported by some other scholars (Chikere *et al.*, 2009). Pure cultures were obtained using four corners streaking on a nutrient Agar petri dishes

Inoculum development from pure cultures

The biomass of each isolate was developed by sub-culturing into peptone broth. The inoculated broth was incubated for 48 hours at 37^{o}C. The biomasses were separated from the broth by cold centrifugation at 5000RPM. This was carried out at the Biotechnology laboratory, Federal Institute of Industrial Research Oshodi (FIIRO), Lagos State.

Preparation of screening medium (Trybutyrin medium) and screening test for lipase- producing bacteria

The hydrolytic activity of isolated bacteria lipase

was done on trybutyrin Agar composed of (g/L): peptone, 10; NaCl, 5; CaCl2.2H2O, 0.1; Trybutyrin selective agaent, 10 mL (v/v). $(NH_4)_2SO_4$ (1.4g), K_2HPO_4 (2.0g), $CaCl_2$ (2.0g), $MgSO_4.7H_2O$ (0.3g), peptone (7.5g), $FeSO_4$ (5.0g), $MnSO_4$ (1.6g), $ZnSO_4$ (1.4g). Out of the total mass generated from each of the isolates, 3 g (wet weight) were weight into the lipase production medium previously autoclaved at standard conditions (Prasad, 2015) and loaded at the shaker incubator at 150RPM for 5 days. On the 5th day, the incubation was stopped, and fermented broth loaded on centrifuge tubes and centrifuged at 5,000g on refrigerated centrifuge (Sigma 4-16). The cell free broths (supernatant) were used for the screening using NaOH (0.5N) titration method.

Lipase screening assay was carried out using olive oil emulsion prepared by emulsifying 25 ml of olive oil with 75 ml of 7% gum Arabic for 10 mins Titration: 2-3 drops of phenolphthalein indicator were added to the reaction mixture and the liberated free fatty acids were titrated with 0.5N NaOH to the end point of pink color at pH 10.0 (Macedo*et al.*, 1997; Lopes *et al.*, 2011; Mendes *et al.*, 2011; Bhavani, *et al.*, 2012; Nagarajan *et al.*, 2014; Ullah *et al.*, 2015). Lipase activity was calculated as micromoles of free fatty acids formed from olive oil per ml of lipase enzyme as given by the equation:

$$\text{Activity} = \frac{(VS - VB).\ N.\ 1000}{S}$$

Where, VS is the volume of 0.05M NaOH solution consumed by the enzyme _substrate cocktail (ml); VB is the volume of 0.05M NaOH solution consumed in the titration by the substrate (control) cocktail; N is the molar strength of the NaOH solution used for titration (0.05M); S is the volume of substrate cocktail solution. One unit of lipase enzyme is defined as the amount of enzyme required to liberate 1µmol of fatty acids from triglycerides (Okoli *et al.*, 2019).

Preparation of Submerged Fermentation Medium for lipase production

This was prepared by weighing (g/l) glutamic acid (0.3g), $(NH_4)_2SO_4$ (1.4g), K_2HPO_4 (2.0g), $CaCl_2$ (2.0g), $MgSO_4.7H_2O$ (0.3g), peptone (7.5g), $FeSO_4$ (5.0g), $MnSO_4$ (1.6g), $ZnSO_4$ (1.4g), Tween 80 (20% v/w), and 1000 ml of distilled water was added and mixed on hot plate magnetic stirrer. Thereafter, NaOH was used to adjust the pH to 5.5 using pH meter (P70 PG Instrument UV model). The medium (150 ml each) was dispensed into 250 ml Erlenmeyer flasks. The flasks and the content were autoclaved at 121 ^{0}C for 15mins.

Biochemical Identification of Isolates

The isolates were identified by colonial morphology, cellular morphology Gram-staining reaction, cellulolytic activity, amylolytic activity, spore test and motility. Biochemical test was carried out thus; catalase test, oxidase test, indole production, methyl red Voges- Proskaeur test (MR VP test), citrate utilization, urease activity, starch Hydrolysis, gelatin, casein hydrolysis, nitrate reduction (NO_3), glucose, sucrose, lactose, galactose, fructose, mannitol, xylose, raffinose, salicin, arabinose and sorbitol. The organisms were subsequently characterized according to Cowan and still manual for the identification of medical bacteria (Barrow and Feltham, 2003).

Molecular identification of isolates

DNA extraction

DNA was extracted using the protocol stated by (Wawrick *et al.*, 2005). Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 °C. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were re-suspended in 520 µl of TE buffer (10) mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 °C and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 °C for 16 h. DNA was collected by centrifugation at 13000 g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

Polymerase chain reaction

PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaqcolourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5′–AGA GTT TGA TCM TGG CTC AG-

3'and - 1525R, 52'-AAGGAGGTGATCCAGCC-32' primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94 °C for 30s, 50 °C for 60s and 72 °C for 1 minute 30 seconds; and a final termination at 72 °C for 10 mins. And chill at 4 °C. GEL (Wawrick *et al.,* 2005: Trindale *et al.,* 2007)

Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, thoroughly by vortexing and keep at -20°C for at least 30 min. Centrifugation for mix 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70% ethanol and mix then centrifuge for 15 min at 7500 g and 4°C. Again remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. then re-suspend with 20 µl of sterile distilled water and kept in -20 °C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nano-drop of model 2000 from thermo scientific.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

Optimization of Temperature during Production of Crude Lipase Enzyme

The determination of the optimum temperature of the enzyme was achieved by incubating the bacterial isolates in a lipase production medium at different temperature ranges of 25-45 °C. The enzyme (Lipase) was diluted conventionally with necessary blanks and control. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source for determination of enzyme activities

Blasting

Blasting was done on the nucleotide blast section of National Centre for biotechnology Information (NCBI)

Optimization of pH Changes during Production of Crude Lipase

The determination of optimum pH of the enzyme production medium was achieved by preparing the lipase production medium hydrolytic activity of isolated bacteria lipase was done on composed of (g/L): peptone, 10; NaCl, 5; CaCl2.2H2O, 0.1; Trybutyrin selective agent, 10 mL (v/v). $(NH_4)_2SO_4$ (1.4g), K_2HPO_4 (2.0g), $CaCl_2$ (2.0g), $MgSO_4.7H_2O$ (0.3g), peptone (7.5g), $FeSO_4$ (5.0g), $MnSO_4$ (1.6g), $ZnSO_4$ (1.4g). The medium was partitioned it Erlenmeyer flasks (250 ml capacity), and the pH of the contents (lipase production medium) of each of the flasks adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0. Out of the total mass generated from each of the isolates, 3grams (wet weight) were weight into the lipase production medium previously autoclaved at standard conditions (Prasad, 2015) and loaded at the shaker incubator at 150RPM for 5 days. there after 5 days of incubation, the Lipase activities were determined using NaOH titration method as previously described above.

Optimization of Nitrogen Sources (Inorganic and Organic) in Lipase Production Medium

The determination of optimum nitrogen of the enzyme production medium was achieved by preparing the lipase production medium hydrolytic activity of isolated bacteria lipase was done on composed of (g/L): peptone, 10; NaCl, 5; $CaCl_2.2H_2O$, 0.1; Trybutyrin selective agent, 10 mL (v/v). $(NH_4)_2SO_4$ (1.4g), K_2HPO_4 (2.0g), $CaCl_2$ (2.0g), $MgSO_4.7H_2O$ (0.3g), peptone (7.5g), $FeSO_4$ (5.0g), $MnSO_4$ (1.6g), $ZnSO_4$ (1.4g). The medium was partitioned in Erlenmeyer flasks (250 ml capacity), and amended with different concentrations of nitrogen sources (NH_4NO_3, KNO_3, Yeast extract). The best pH observed during the optimization for pHwas used to adjust all medium during optimization for nitrogen source, and loaded at the shaker incubator at 150 rpp for 5 days there after 5 days of incubation; the Lipase activities were determined using NaOH titration method as

previously described above.

Optimization of Carbon Source in Lipase production medium

The determination of optimum carbon Lipase production medium hydrolytic activity of isolated bacteria lipase was done on composed of (g/L): peptone, 10; NaCl, 5; $CaCl_2.2H_2O$, 0.1; Trybutyrin selective agent, 10 mL (v/v). $(NH\text{-}_4)_2SO_4$ (1.4g), K_2HPO_4 (2.0g), $CaCl_2$ (2.0g), $MgSO_4.7H_2O$ (0.3g), peptone (7.5g), $FeSO_4$ (5.0g), $MnSO_4$ (1.6g), $ZnSO_4$ (1.4g). Influence of various carbon sources present in fermentation on enzyme production was studied by using different carbon sources such as Tobacco seed oil (5 ml/100 ml of production medium), Tobacco seed oil (10 ml /100 ml of production medium), Olive oil (5 ml/100 ml of production medium), Olive oil (10ml /100 ml of production medium). The Erlenmeyer flasks were loaded at the shaker incubator at 150RPM for 5 days there after 5 days of incubation; the Lipase activities were determined using NaOH titration method as previously described above

RESULTS AND DISCUSSION

The results of the Lipolytic potentials of wild strains of bacterial isolates from different soil in environment in Abeokuta were reported in Table 1. Out of a total of eleven (11) isolates, isolates coded H, A, B, F had the highest lipase producing potentials as their lipolytic activities were 21.0 units, 14.0 units, 12.0 units, and 13.0 units, respectively (Table 1). Thus out of a total of 11 bacterial Isolates Subjected under Lipase – Screening, only 4 Isolates (H, A, B, and F) showed the highest abilities to produce lipase. In comparison with related studies, across the globe, *Acinetobacter* species isolated from Oil – contaminated Soil in South Korea, showed different degrees of lipolytic activities using Spirit blue Agar and Rhoda Nine B – agar (Anbu *et al.* 2011). In addition, Aifuwa *et al.* (2017) reported that fungi such as *Penicillium* sp, *Mucor* sp, *Lasioplodia* sp, and *Trichoderma* sp, showed about 0.063 unit, 0.284 unit, 0.142 units, 0.022 units, and 0.022 units, respectively, and this is comparable with the observation in this current study. The isolation of Lipase– producing microorganisms from oil – impacted soil within Ogun state-Nigeria is not surprising as the presence of oil in the environment mount enormous biological stress in an environment which leads to the acquisition of lipid-degrading genus within bacterial fungi in the environment.

Table 1. Screening for lipase producing potential

Sl. N.	Bacterial isolates Refcode	Average TITRE (ml)	Activity (Unit) ±SD
1	A1	0.70	14.00 ± 0.021^{a}
2	B1	0.60	12.00 ± 0.115^{b}
3	C1	0.60	12.0 ± 0.073^{b}
4	D1	0.60	12.00 ± 0.023^{b}
5	E1	0.55	11.00 ± 0211^{b}
6	F1	0.65	13.00 ± 0.321^{b}
7	G1	0.53	10.50 ± 0.117^{c}
8	H1	1.05	21.00 ± 0.213^{ab}
9	I1	0.60	12.00 ± 0.116^{b}
10	K1	0.55	11.00 ± 0.112^{b}
11	L1	0.60	12.00 ± 0.074^{b}

Numbers with different superscripts are statistically significant at p- values >0.05 (Duncan multiple range Test)

This Lipid – degrading genes expresses its function of lipid degradation through release of Lipolytic enzymes (Baruwa *et al.,* 2019) Okoro and Chuma (2018) also reported the presence of Lipase hyper – producing *Lactobacillus* species from Nigeria Local food stuffs.

The identities of these 4 hyper – producing bacteria were determined using biochemical tests (Table 2). The bacterial isolates H, A, B and F as best hyper – producers were identified as *Bacillus brevis*, *Acinetobacter mallei*, *Bacillus megaterium*, and *Bacilluspolymyxa* (Table 2). The identities of the hyper – producing strains were further identified using molecular biology tools (Polymerase chain Reaction and sequencing).

The Bacterial isolates H, A, B, and F were molecularly identified as *Brevibacillus brevis* strain HK 544, *Pseudomonas aeruginosa* strain WES2, *Bacillus megaterium* strain WH13, and *Bacillus Subtilis* strain BS 01, respectively (Table 3).

The abilities or potentials to require Lipase – producing genes among microorganisms cuts across different physiological groups, and genera of Micro-organism. Aifuwa *et al.* (2017) reported high lipolytic potentials amongst wild type strains of *Curvularia*, *Lasiodiplodia*, *Trichoderma* and *Penicillum*. Okoli *et al.* (2018) had previously reported the production of lipase from thermo-tolerant *Bacillus* species strains. Jaiswal *et al.* (2017) also in a similar trend reported high lipolytic potentials within *Pseudomonas* stutzeri, *Bacillus coagulans*, *Proteus mirabilis*, and Bacillus sporothermo-durans (thermophilic).

Isolate code/ our reference	Gram Reaction	Sugar / substrate fermentation														Most Probable Identity
		Catalase	Oxidase	Urease	Casein hydrolysis	Gelatin hydrolysis	Starch hydrolysis	Nitrate reduction	Galactose	Glucose	Sucrose	Lactose	Arabinose	Sorbitol	Salicin	Ribose	Maltose	Mannitol	Fructose	
A	Gram negative rods	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	*Acinetobacter mallei*
B	Gram positive rods	-	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	+	*Bacillus megaterium*
F	Gram positive rods	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	*Bacillus polymyxa*
H	Gram positive rods	+	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-	-	+	*Bacillus brevis*

Optimization for Cultural Conditions

The optimization of bacterial production of Lipase was independently carried out using variation in pH of culture/production media. The four (4) bacterial Isolates showed different trend of Lipase production when subjected to different PH conditions between pH 5.0 (acidic) to pH 9.0 (alkaline). *Brevibacillus brevis* strain HK 544 (referenced/coded Isolate H) showed optimum/maximum production potential for Lipase at PH 8.0 where the Lipase activities recorded was 26.0 units. In addition, the optimum PH for lipase production in Isolate A (*Pseudomonas aeruginosa*), Isolate B (*Bacillus megaterium* strain WH13) and Isolate F (*Bacillus subtilis* BS 01) were pH 7.0 (28.0 units of lipase), pH 7.0 (22.0 units) pH 9.0 (16.0 units), respectively (Figure 1). It is worthy to mention that preference to a particular pH in order to release either primary or secondary Metabolites is a gene – control physiological process.

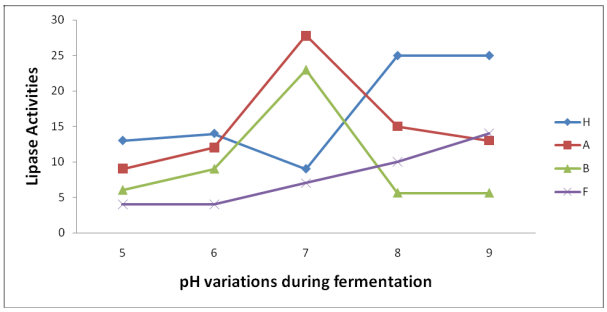

Fig. 1. Effect of pH on lipase activities

For – instance while the present study showed that Isolates H, A, B and F can optimally produce Lipases at pH values of 8.0, 7.0, 7.5 and 9.0, respectively, Hassan *et al.*, (2006) reported that *Bacillus* sp. FH5 showed optimal Lipase producing potential at PH 8.0 (meaning Lipase of *Bacillus* sp. FH5 is an Alkaline Lipase).

In another related investigation, Kareem *et al*, (2017). *Bacillus thuringiensis* and *Lysinibacillus sphaericus* is pH 7.0 (an indication that both Lipases are neutral Lipases).

Changes or variations in temperature had different impact on the abilities of the hypes – producing bacterial to produce Lipase. In Isolate H (*Brevibacterium brevis* strain HK 544), at temperatures between 25^0 to 40 ^oC the activities of Lipase from *Brevibacterium brevis* had repressed activities between 6.0 units to 8.0 units.

The optimum temperature for production of Lipase by *Brevibacterium brevis* (Isolate H) was

observed to be at 45^{0}C where Lipase units of 28.0 units were recorded (Figure 2). In addition, temperatures between 25^{0}C - 40 ^{o}C, the *Pseudomonas aeruginosa* strain WES2 (Isolate A), had lower Lipase activities of 8.0 – 11.0 units. However, the activities of *Pseudomonas aeruginosa* showed significant increase to 22.0 units at temperature of 45 ^{o}C.

Considering Isolate B which was identified to be *Bacillus megaterium* strain WH 13 the temperature of 25^{O}C – 37^{O}C was not favorable to the bacterial Lipase – producing potential as lipase activities were only observed to be between 5.0 to 10.0 units.

However, at the optimum temperature for production of lipase by isolate B (*Bacillus megaterium* was 45 ^{o}C as the Lipase activity recorded for Isolate B Lipase at 45 ^{o}C was significantly higher (28.0 units) when compared to the Lipase activities at temperature at temperatures of 25 ^{o}C – 40 ^{o}C (Fig. 2).

Furthermore, the Lipase of Isolate F (*Bacillus subtilis*) showed lower activities at temperature of 25^{o}C (14.0 units), and 40 ^{o}C (8.0 units) (Figure 2) in addition two option temperature of 37^{o}C and 45^{o}C where the Lipase activities of 24.0 units and 26.0) units were recorded, respectively (Figure 2). The trend of observation in this current study showed that most of the Lipases from the difference bacterial isolate are within mesophilic temperature ranges.

Hassan *et al.* (2036) observed that the Lipase of Bacillus sp. FH5 is must active at a cultural temperature 37^{o}C.

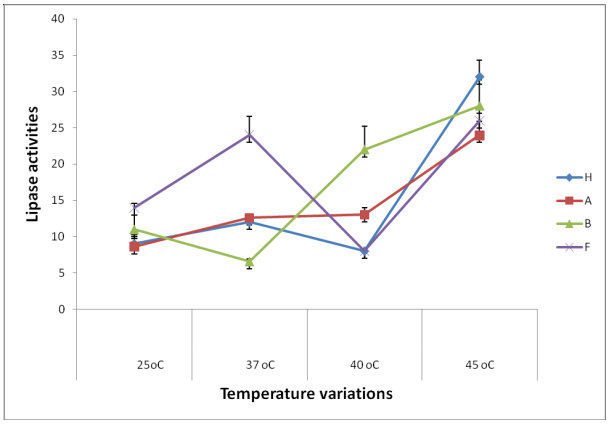

Fig. 2. Effect of temperature on lipase activities

The effect of variations in the concentration of two carbon sources (Tobacco seed oil (TSO), and Olive oil were studies and reported Figures 3. Observation showed that for Isolate H (*Brevibacillus brevis*), the higher the concentration of both tobacco Seed oil and olive oil (from 5 ml /100m f production of Lipase of Isolate H in the instance where 5ml of tobacco seed oil and olive oil were used, the Lipase activities were 110 units and 8.0 units respectively (Figure 3.0). In addition, at carbon source level of 10 ml/100 m/medium, the Lipase activities of 180 units, and 42.0 units were observed in Tobacco seed oil medium, and olive oil amended medium. This significant difference in activities of Isolate H when using Tobacco seed oil and olive oil showed that the bacterial may have developed better preference to olive oil than tobacco seed oil. In another consideration, the fact that olive is more readily oxidisable than tobacco seed oil may be an a good justification for Lipase activities of Isolate H under olive as carbon sources higher than Lipase activities when Tobacco seed oil was utilized in process (Figures 3).

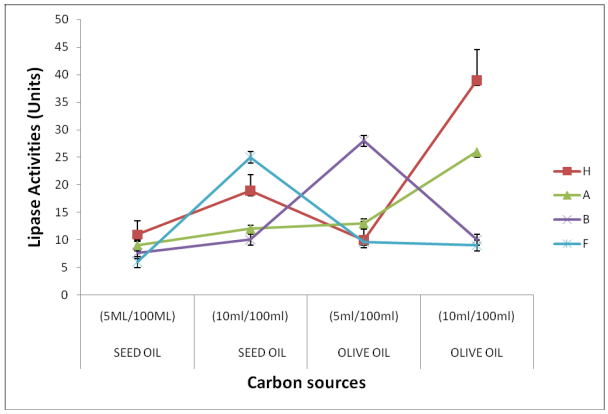

Fig. 3. Effect of carbon source variation on lipase activities

In the similar fashion /trend, Isolate A, F showed higher activities of Lipase of Production Medium with showed different trend in olive amended medium as lower concentration of 5m/100m/had higher activities of Lipase (28.0 units) when compared to the production option amended with

Table 3. Summary of Molecular Identity of some selected lipase hyper-producing strains of Bacteria

Sl.N	Isolate codes	IDENTITY	% Similarity	Accession Number
1	H	*Bevibacillus brevis* strain HK 544	99.44	CPO42161.1
2	A	*Pseudomonas aeruginosa* WES2	99.93	MN960116.1
3	B	*Bacillus megaterium* strain WH13	100	Mn372086.1
4	F	*Bacillus subtilis* BS 01	100	MT372489.1

10ml/a00m of olive oil where Lipase activity was observed to be 10.0 units (Figure 3.0) Hassan *et al* (2006) utilized simple sugars as inducible carbons and observed that the Lipase of *Bacillus* sp FH5 is most active on incorporation into growth medium.

Prasasty *et al.,* (2016) report that *Bacillus* as ad *Pseudomonas alcaligenes* isolated from Plateau Soil West Java, Indmesia were grown in media containing olive oil as sole carbon source. The report by Prasasty *et al.,* (2016) showed that olive oil was optimal carbon source for Lipase (*Bacillus* and *Pseudomonas*).

In a related study, Moses *et al.,* (2022) demonstrated that *Klebsiella Pneumoniae* grown on vegetable oil released extracellular Lipase that is comparatively high in terms of enzyme activity. In this current study, it is worthy to mention that the use of Tobacco Seed oil (TS) still remain novel for use as carbon sources in microbial enzyme production. The nitrogen sources varied during the microbial response optimization and were ammonium nitrate, Potassium nitrate, and yeast extract. In Isolate H (*Brevibacillus brevis* strain HK544), higher level of NH_4NO_3 (6 g/l) lead to higher activity of Lipase of Isolate H (35.0 units). In addition both 6g/L KNO_3 showed no significant difference in Lipase activities of Isolate H. In a different fashion, the Lipase of Isolate H showed slightly higher activities in the presence of lower concentration of yeas Extract as 3 g/l yeast extract in Production medium lead to higher activity of Lipase (11.0 units) which is higher the activities of Lipase at 6.0 g/l was observed to the 9.0 units (Figures 4). Isolates A, and B in a similar direction responded better on exposure to higher concentration of nitrogen sources 6 g/l NH_3NO_3 impacted higher Lipase activities than the lower concentration of nitrogen sources (3 g/l of NH_4NO_3, $KN0_3$ and yeast extract (Figure 4).

In Isolate F (*Bacillus subtilis* strain BS01) consistently then the bacterial isolate responded better to lower concentrations of nitrogen sources used. The activities of *Bacillus subtilis* lipase at 3g/l NH_3NO_3, 3 g/l KNO_3 and 3 g/l yeast extracts were 7.5 units, 26.0 units, and 8.0 units, respectively (Figure 4). In comparison with amendment of production medium with 6 g/l NH_4N0_3, 6 g/l KNO_3, and 6 g/L yeast extract, the activities of isolates F lipase were observed to be 6.0 units, 15.0 units, and 8.0 units, respectively (Figure 4).

Mazhar *et al.,* (2017) opined that the use of inorganic nitrogen sources such as (NH4)2 $S0_4$,

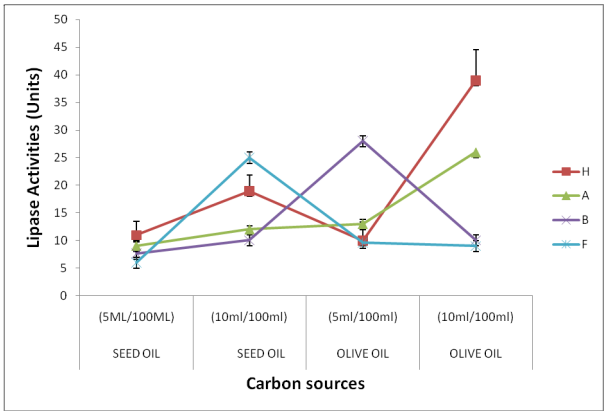

Fig. 4. Effect of nitrogen source variation on lipase activities

NH_4NO_3, NH_4Cl showed that the best nitrogen source for *Bacillus Subtilis* PCSIRNL-39 was NH_4N0_3 (45.0 units of lipase activity.)

CONCLUSION

The use of lesser known oil-based carbon source (Tobacco Seed Oil) was employed for Lipase production using 4 different bacterial isolates. The results of this Prospective investigation showed the different Bacterial isolates responded very well to tobacco seed oil as sole carbon source for Lipase production. Further investigation could be required in terms of scale- up for industrial scales using design of equipment and Engineering models.

REFERENCES

Akimoto, M., Nagashima, Y. and Sato, D. 1999. A kinetic study on lipase-catalyzed interesterificaiton of soybean oil with oleic acid in a continuous packed bed reactor. *Appl Biochem Biotechnol.* 81 : 131-142.

Ambu, P., Noth, M.J., Kim, D.H., Seo, J.S., Hur, B.K. and Kyeong, H.M. 2011. Screening and optimization of extracellular Lipases by *Acinetobacter* species isolated from oil contaminated soil in South Korea. *African Journal of Biotechnology.* 10(20) : 4147-4156.

Andersson, R.E., Hedlund, C.B. and Jonsson, U. 1979. Thermal inactivation of a heat resistant lipase produced by the psychrotrophic bacterium *Pseudomonas fluorescens*. *J. Dairy Sci.* 62 : 361-367.

Bhavani, M., Chowdary, G.V., David, M. and Archana, G. 2012. Screening isolation and biochemical characterization of novel lipase producing bacteria from soil samples. *Int. J. Biol. Engr.* 2(2): 18-22.

Cordova, J., Neummaoui, M., Ismali, M., Morin, A., Roussos, S., Raimbault, M. and Benjilai, B. 1998. Lipase production by solid state fermentation of olive

cake and sugar cane bagasse. *J. Mol. Catal. B Enzym.* 5(1- 4) : 75-78.

Godfrey, T. and West, S. 1996. Introduction to industrial enzymology, in Industrial enzymology, pp1-8.

Gupta, J.K. and Soni, S.K. 2000. Industrial uses of enzymes. *Journal of Punjab Academy of Sciences.* 2 : 75-80.

Jaiswal, A., Preet., M. and Tripti, B. 2017. Production and optimization of lipase enzyme from mesophiles and thermophiles. *Journal of Microbial & Biochemical Technology.* 9(3) : 126-131.

Javed, S., Azeem, F., Hussain, S., Rasul, I., Siddique, H. M., Riaz, M. Afzal, A. Kouser, A. and Nadeem, H. 2017. Bacterial Lipases: A Review on Purification and Characterization. *Progress in Biophysics and Molecular Biology.* 132 : 23-34.

Khan, I. M., Dill, C. W., Chandan, R. C. 1967. Production and properties of extracellular lipase of a Chromobacterlipolyticum. *Biochim. Biophys. Acta.* 132: 68-77.

Lopes, D.B., Fraga, L.P., Fleuri, L.F. and Macedo, G.A. 2011. Lipase and esterase – to what extent can this classification be applied correctly? *Current Technol. Aliment.* 31(3) : 608-613.

Macedo, G.A., Lozano, M.M.S. and Pastore, G.M. 2003. Enzymatic synthesis of short chain citronellyl esters by a new lipase from Rhizopussp. *J. Biotechnol.* 6 : 72-75.

Mazhar, H., Abbas, N., Ali, S., Sohail, A., Hussain, Z. and Ali, S.S. 2017. Optimized production of Lipase from *Bacillus subtilis* PCSIRNL-39. *African Journal of Biotechnology*. 16(19) : 1106-1115.

Moses, B.E., Egwim, C.E., Abdulkadir, A., Liman, M.L., Alex, V.A. and Manya, Z. 2020. Production, Partial Purification and characterization of lipase enzyme expressed by *Klebsiella pnuemoniae* of vegetable oil contaminated soil. *International Journal of Biochemistry and Biophysics.* 8(2) : 30-39.

Musa, H. and Adebayo-Tayo, B.C. 2012. Screening of microorganisms isolated from different Envirnmental samples for Extracellular Lipase Production, *AU.J.T.* 15(3) : 179-186.

Nagarajan, J., Nawawi, N.M. and Ibrahim, A.L. 2014. Characterization of lipase producing *Rhodococcus* sp. from peninsular Malaysia. *J. Life Sci. Technol.* 2(1): 12-19.

Okoli, C.B., Orji, F.A. and Lawal, A.K. 2019. Isolation, Screening, and Optimization of *Bacillus* spp for lipolytic enzyme production. *Journal of Industrial Research and Technology.* 7 (1): 112-129.

Pallavi1, P., Ravikumar, T. and Reddy, R.S. 2015. Isolation and characterization of lipase producing bacteria for biodegradation of oil contamination. *International Journal of Current Research and Academic Review*. 3(8): 55-66.

Svendsen, A. 1994. Sequence comparisons within the lipase family in Lipases: Their structure. *Biochemistry and Applications.* 1-21.

Ullah, N., Daud, M., Hidayatullah, Shabir, M. and Ozkan, A.T. 2015. Screening identification and characterization of lipase producing soil bacteria from upper dir and Mardan Khyber Pakhtunkhwa. *Pakistan. IJB* 6(2) : 49-55.